Identification of single nucleotide polymorphisms of the PI3K-AKT-mTOR pathway as a risk factor of central nervous system metastasis in metastatic breast cancer

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Abstract: Introduction: The PI3K-AKT-mTOR pathway may be involved in the development of CNS metastasis from breast cancer. Accordingly, here we explored whether single nucleotide polymorphisms (SNP) of this pathway are associated with altered risk of CNS metastasis formation in metastatic breast cancer patients.
Methods: The GENEOM study (NCT00959556) included blood sample collection from breast cancer patients treated in the neoadjuvant, adjuvant or metastatic setting. We identified patients with CNS metastases for comparison with patients without CNS metastasis, defined as absence of neurological symptoms or normal brain MRI before death or during 5-year follow-up. Eighty-eight SNP of PI3K-AKT-mTOR pathway genes were selected for analysis: AKT1 (17 SNP), AKT2 (4), FGFR1 (2), mTOR (7), PDK1 (4), PI3KR1 (11), PI3KCA (20), PTEN (17), RPS6KB1 (6).
Results: Of 342 patients with metastases, 207 fulfilled the inclusion criteria: 107 patients remained free of CNS metastases at last follow-up or date of death whereas 100 patients developed CNS metastases. Among clinical parameters, hormonal and HER2 status as well as tumor vascular emboli were associated with risk of CNS metastasis. Only PI3KR1-rs706716 was associated with CNS metastasis in univariate analysis after Bonferroni correction ($p<0.00085$). Multivariate analysis showed associations between AKT1-rs3803304, AKT2-rs3730050, PDK1-rs11686903 and PI3KR1-rs706716 and CNS metastasis.
Conclusion: PI3KR1-rs706716 may be associated with CNS metastasis in metastatic breast cancer patients and could be included in a predictive composite score to detect early CNS metastasis irrespective of breast cancer subtype.

Cover Letter

Lille, 29 September 2017

Dear Editors,

Please find enclosed a revised version of our manuscript entitled "Identification of single nucleotide polymorphisms of the PI3K-AKT-mTOR pathway as a risk factor of central nervous system metastasis in metastatic breast cancer" which we hope is now acceptable for publication in the *European Journal of Cancer*.

We would like to thank you and the reviewer for the constructive comments. Our point-to-point response is listed below and changes are accordingly highlighted in the manuscript.

Thank you very much for your interest in our work.

Kind regards

Emilie Le Rhun, MD, PhD

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IDENTIFICATION OF SINGLE NUCLEOTIDE POLYMORPHISMS OF THE PI3K-AKT-mTOR PATHWAY AS A RISK FACTOR OF CENTRAL NERVOUS SYSTEM METASTASIS IN METASTATIC BREAST CANCER

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"I confirm that all the authors have made a significant contribution to this manuscript, have seen and approved the final manuscript, and have agreed to its submission to the *European Journal of Cancer*".

Signed (corresponding author): .....
Date: 01 AUG 2017

*Revision Notes

Response to Reviewer'Comments

Reviewer 2:
This manuscript by Le Rhun et al. is a relevant contribution to the field: with respect to brain metastasis biology, and even more so with respect to identification of predictive factors (that are currently lacking, making selection of patients for e.g. preventive measures difficult). In principle, the language is comprehensible, the data appears sound, the statistics valid; some parts of the discussion can be improved.
I have some points that need to be addressed before publication can be recommended:

1. Please provide a more in-depth discussion of what is known about the biological relevance of the PI3K/MTOR pathway in brain metastasis of breast cancer and other entities.

RESPONSE
This has been done although with the consideration of the space limitations (see page 4), including two more references.

"The PI3K-AKT-mTOR pathway controls cell cycle, survival, differentiation, proliferation, motility, metabolism, and genomic stability and may be the most frequently activated pathway in human cancer [19,20,21]. Moreover, it also regulates the behavior of normal cells and contributes to host cell tumor cell interactions, e.g., during angiogenesis and inflammation [21-27]. PI3K-AKT-mTOR pathway genetic lesions are frequent in breast cancer and may mediate resistance to HER2-targeted agents and hormonal agents [28]. Activation of the PI3K pathway has specifically been observed in brain metastases from breast cancer, regardless of subtype as defined by hormone receptor or HER2 status [29,30], potentially mediated by the loss of PTEN expression as demonstrated in paired primary tumor and brain metastasis samples [31]. In fact, the loss of PTEN may directly promote brain invasiveness of metastatic breast cancer cells [27]. Here we sought to identify SNP of the PI3K-AKT-mTOR pathway associated with increased risk of CNS metastasis formation in patients with metastatic breast cancer."

Nosaka R, Yamasaki F, Saito T, Takayasu T, Kolakshyapati M, Amatya VJ, et al. Role for loss of nuclear PTEN in a harbinger of brain metastases. J Clin Neurosci Off J Neurosurg Soc Australas 2017;44:148–54.

Hohensee I, Lamszus K, Riethdorf S, Meyer-Staeckling S, Glatzel M, Matschke J, et al. Frequent genetic alterations in EGFR- and HER2-driven pathways in breast cancer brain metastases. Am J Pathol 2013;183:83–95.

2. In Fig. 3, please provide the cumulative incidence of brain met formation for the combined score of the 4 SNIPs.

RESPONSE

As requested by the Referee, we introduced a new Figure 3E including data on the cumulative incidence of brain metastases for the combined score. To this end, we classified the patients by the absence or presence of 0 versus 1 versus 2 risk genotypes.

3. It would greatly increase the value of the study if the 4 SNIP / 1 SNIP signature would be confirmed in an independent dataset - however, I see that this might be quite labor intensive and potentially beyond the scope of the study. Would a more focussed addition of data in this respect possible?

RESPONSE
We acknowledge the weakness of the lack of a validation cohort, as already stated in the Discussion. We appreciate the Referee`s understanding that such data are very difficult to obtain, notably within the time frame of this revision. Please note that this would require a patient population with sufficiently long follow-up where blood samples were obtained, too.

4. The finding of the "tumor vascular emboli" / "peritumoral emobli" association with BM occurence is potentially highly interesting, potentially supporting the long-standing idea that the coagulation system is linked to metastasis formation. However, the authors fail to explain what they actually mean by these "emboli": how they defined and measured this parameter.

RESPONSE
Thank you for this comment. Emboli are defined here as the presence of tumor cells in the vessels of the surgical samples. No scores and no cut-offs were used to define this value. This information has been added to supplementary note 1.

"The following demographic and clinical data were collected: age at diagnosis of metastasis, histological type of breast cancer, tumor differentiation, Scarff Bloom Richardson grade modified by Elston and Ellis, mitotic activity measured by Ki67 labeling, hormone receptor status, HER2 status, peritumoral emboli defined as the as the presence of tumor cells in the blood vessels of the resected tumor specimens, initial treatment of breast cancer, time interval between diagnosis of breast cancer and diagnosis of first metastasis, sites of metastasis, number of lines of treatment for metastatic disease, specific treatments for CNS metastasis, date of death, and cause of death. Estrogen and progesterone receptors were defined as positive when more than 10% of nuclei were positive by immunohistochemistry. HER2 was defined as positive when the immunohistochemistry score was 3+, or when it was 2+ and the tissue was positive by chromogenic *in situ* hybridization (CISH) or fluorescence *in situ* hybridization (FISH)."

Highlights

- Early diagnosis of CNS metastasis may improve outcome of breast cancer patients.
- Identification of CNS metastasis risk may influence imaging follow-up and help to design prevention strategies.
- PI3K-AKT-mTOR pathway SNP may identify metastatic breast cancer patients at risk of CNS metastasis.

European Journal of Cancer

Identification of single nucleotide polymorphisms of the PI3K-AKT-mTOR pathway as a risk factor of central nervous system metastasis in metastatic breast cancer

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Abstract

Introduction: The PI3K-AKT-mTOR pathway may be involved in the development of CNS metastasis from breast cancer. Accordingly, here we explored whether single nucleotide polymorphisms (SNP) of this pathway are associated with altered risk of CNS metastasis formation in metastatic breast cancer patients.

Methods: The GENEOM study (NCT00959556) included blood sample collection from breast cancer patients treated in the neoadjuvant, adjuvant or metastatic setting. We identified patients with CNS metastases for comparison with patients without CNS metastasis, defined as absence of neurological symptoms or normal brain MRI before death or during 5-year follow-up. Eighty-eight SNP of PI3K-AKT-mTOR pathway genes were selected for analysis: AKT1 (17 SNP), AKT2 (4), FGFR1 (2), mTOR (7), PDK1 (4), PI3KR1 (11), PI3KCA (20), PTEN (17), RPS6KB1 (6).

Results: Of 342 patients with metastases, 207 fulfilled the inclusion criteria: 107 patients remained free of CNS metastases at last follow-up or date of death whereas 100 patients developed CNS metastases. Among clinical parameters, hormonal and HER2 status as well as tumor vascular emboli were associated with risk of CNS metastasis. Only PI3KR1-rs706716 was associated with CNS metastasis in univariate analysis after Bonferroni correction ($p<0.00085$). Multivariate analysis showed associations between AKT1-rs3803304, AKT2-rs3730050, PDK1-rs11686903 and PI3KR1-rs706716 and CNS metastasis.

Conclusion: PI3KR1-rs706716 may be associated with CNS metastasis in metastatic breast cancer patients and could be included in a predictive composite score to detect early CNS metastasis irrespective of breast cancer subtype.

Keywords

SNP, PTEN, brain, cerebral, leptomeningeal, prediction, genetic, predisposition, prevention

Text

INTRODUCTION

Breast cancer represents the most common cancer in women. Central nervous system (CNS) metastases occur in up to 10% of patients [1] and herald poor outcome: survival varies from 2.7 to 26.8 months with solid brain metastases, by breast cancer subtype [2] and is 4 months with leptomeningeal metastases [3-6]. Treatment of CNS metastasis aims not only for prolonging survival, but also at prevention or delay of neurological deterioration [7].

The identification of patients at risk could help to increase the efficacy of treatment of CNS metastasis. While cerebrospinal imaging is not part of standard follow-up in patients without neurological signs, the identification of subgroups of patients at risk could allow the implementation of more intensive follow-up and early intervention strategies.

Brain metastases risk is increased in triple-negative and human epidermal growth factor receptor (HER) 2-positive tumors [1, 8-14]. Risk factors for leptomeningeal metastases include opening of the ventricular system during surgery for solid brain metastases and resection of cerebellar metastases [15,16] and breast cancer patients specifically lobular subtype and triple-negative tumors [4,5,6,17].

Genetic variations could also help to define populations at risk. Single nucleotide polymorphisms (SNP) represent the most frequent type of variations of the human

genome [18]: they represent a single nucleotide variation at a specific position in the genome present at a frequency of 1%-50% in the general population that is maintained through heredity. While not causing disease, SNP can modify protein structure and function and thereby influence susceptibility to disease, including cancer [18].

The PI3K-AKT-mTOR pathway controls cell cycle, survival, differentiation, proliferation, motility, metabolism, and genomic stability and may be the most frequently activated pathway in human cancer [19,20,21]. Moreover, it also regulates the behavior of normal cells and contributes to host cell tumor cell interactions, e.g., during angiogenesis and inflammation [21-27]. PI3K-AKT-mTOR pathway genetic lesions are frequent in breast cancer and may mediate resistance to HER2-targeted agents and hormonal agents [28]. Activation of the PI3K pathway has specifically been observed in brain metastases from breast cancer, regardless of subtype as defined by hormone receptor or HER2 status [29,30], potentially mediated by the loss of PTEN expression as demonstrated in paired primary tumor and brain metastasis samples [31]. In fact, the loss of PTEN may directly promote brain invasiveness of metastatic breast cancer cells [27]. Here we sought to identify SNP of the PI3K-AKT-mTOR pathway associated with increased risk of CNS metastasis formation in patients with metastatic breast cancer.

MATERIALS AND METHODS

Patients

We conducted a secondary analysis in a subpopulation of patients from the GENEOM study (NCT00959556) that aimed at identifying constitutional genetic

variants predictive of response to chemotherapy and hormone therapy in adult patients with histologically confirmed breast cancer and included 914 women between November 2007 and January 2012. Our aim was to identify biomarkers of CNS metastasis risk among patients who developed metastases, at diagnosis of breast cancer or during follow-up (n=342) (Supplementary Note 1). Among patients fulfilling the inclusion criteria (n=207), CNS metastases were diagnosed at initial diagnosis of breast cancer or during follow-up, on brain MRI (n=87, 87%) or cranial CT (n=13, 13%). Histological confirmation was obtained in 10 brain metastasis patients. Leptomeningeal metastasis was defined by the presence of tumor cells in the cerebrospinal fluid (CSF) (n=25) or by characteristic MRI findings in a patient presenting with clinical signs suggestive of leptomeningeal metastases in the absence of positive CSF cytology (n=15). In 9 patients, the diagnosis was based on clinical evaluation and brain imaging only. Patients with extra-CNS metastasis only were defined by the absence of neurological symptoms or signs or normal brain MRI before death or during at least 5 years of follow-up after the diagnosis of the first metastasis. Patients with neurological symptoms of unclear origin, unclear cause of death, or follow-up below 5 years after the diagnosis of the first metastasis were excluded.

SNP selection

SNP of the PI3K-AKT-mTOR pathway were selected based on a systematic literature search. Eligible SNP had to have a minor allele frequency ≥ 0.05 in a European population, based on a 1000 genomes database (https://phase3browser.1000genomes.org/index.html). Eighty-eight SNP of the PI3K-AKT-mTOR pathway were finally considered for the genomic analysis. Two were

excluded (AKT1 rs3803304 and AKT1-rs2498786) due to a discordance between frequencies observed in our population and the database. A total of 86 SNP was finally analyzed (Supplementary Table 1). Details on genomic analyses and SNP studies are provided in Supplementary Note 2.

Statistical analysis

Overall survival (OS) was defined as time interval from diagnosis until death from any cause using the Kaplan-Meier method. Patients alive were censored at last follow-up. Cumulative incidence of CNS metastases was estimated using a competing risk approach considering the time interval from diagnosis of first metastasis to the date of diagnosis of CNS metastases, with death without CNS metastases considered as a competing event; patients alive without CNS metastases were censored at the date of last follow-up. Associations between CNS metastases and clinical parameters and genomic parameters were evaluated using sub-distribution hazard ratios estimated in Fine and Gray regression models. The first step consisted in the analysis of clinical factors. A multivariate competing risk regression model was performed for parameters significantly associated with CNS metastasis in univariate analysis ($p<0.05$).

A similar type of modeling was used for the second step of the analysis, evaluating the association of genomic parameters with the risk of CNS metastases. A SNP was considered as evaluable after verification of comparison for genotypic frequencies according to the Hardy-Weinberg equilibrium. SNP were excluded if the minor allele frequency was below 1%, or if genotyping analyses were performed in less than 90% of the patients of the cohort. Patients with less than 90% of SNP analyzed were excluded. For each SNP, the analysis was performed considering a dominant model

(dominant genotype versus others), recessive model (recessive genotype versus others) or log-additive model (3 ordered genotypic classes). The significance level was set to $p<0.05$, and a Bonferroni correction for multiple testing was also determined according to the number of independent SNP evaluated (threshold=0.05/number of independent SNP with $r^2<0.8$). SNP significantly associated with CNS metastasis in univariate analysis (using level $p<0.05$) were included in a multivariate competitive risks regression model including a step by step selection procedure of variables. Akaike information criterion (AIC) was applied for the selection of the appropriate SNP coding when several modellings were associated with CNS metastasis. A composite score was also computed from the estimated regression coefficients of the multivariate model including all SNP significant at a 5%-significant level. Lastly, the association of this genomic score with the risk of CNS metastases was evaluated in multivariate analysis adjusted for clinical parameters significantly linked to CNS metastasis. Confidence intervals were re-estimated using a boostrap approach with 1000 samplings. Harrell C discrimination index was computed. Statistical analyses were performed using Stata software (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP) and SNPassoc package of R software v3.3.1.

RESULTS

Clinical patient characteristics

Among the 914 patients enrolled in GENEOM, 342 patients had metastatic disease, 119 at diagnosis of breast cancer and 223 during follow-up. Among these 342 patients, 135 patients were excluded, leading to a study population of 207 patients (Figure 1). The median follow-up of this cohort is 9.1 years (range 4.8-10.5) after the

diagnosis of first metastasis. Overall, 107 patients remained free of CNS metastases at last follow-up or date of death whereas 100 patients developed CNS metastases. Median time interval between first diagnosis of metastasis and first diagnosis of brain metastasis was 1.4 years (0-8.4); it was 2.1 years between first diagnosis of metastasis and first diagnosis of leptomeningeal metastasis. CNS metastasis was the first metastatic site in 8 patients (8%). Treatment of brain metastases included surgery in 10, stereotactic radiotherapy in 14, whole brain radiotherapy in 51, and pharmacotherapy in 53 patients. Treatment of leptomeningeal disease included intra-cerebrospinal fluid pharmacotherapy in 28, focal radiotherapy in 5 and whole brain radiotherapy in 1, and systemic pharmacotherapy in 29 patients. At last follow-up, 189 patients had died. Median overall survival was 5.3 years (95% CI 4.5-6.1) since breast cancer diagnosis and 2.8 years (95% CI 2.3-3.3) since first diagnosis of metastasis. Median overall survival after diagnosis of CNS metastasis was 4.7 months (95% CI: 3.6-6.4) for brain metastases patients and 4 months (95% CI: 2.3-4.9) for leptomeningeal metastases patients (Figure 2A,B,C).

Patient characteristics and association of clinical characteristics with risk of CNS metastases are summarized in Table 1. Median age at breast cancer diagnosis was 50 years (range, 22 to 79); age was not associated with occurrence of CNS metastasis. A lower time interval between breast cancer diagnosis and diagnosis of first metastasis was associated with an increased risk of CNS metastases whereas histology and Scarff Bloom Richardson scores were not. Cumulative incidence of CNS metastases was higher in patients with hormone receptor-positive and HER2-positive or triple-negative tumors, as well as in patients with peritumoral emboli on univariate and multivariate analysis (Figure 2D,E). Initial treatment was not associated to the occurrence of CNS metastases (Supplementary Table 2).

SNP analyses

Of 86 SNP, 3 were excluded from analysis: RPS56KB1-rs1292033 for not respecting the Hardy Weinberg equilibrium, and PI3KCA-rs17849071 and PI3KCA-rs7641889 because 10.1% of patients could not be genotyped for these 2 SNP. No SNP had a minority allele frequency below 1%. The genotype of all patients was analyzed for more than 90% of SNP, and thus all patients were included in this analysis. Thus 83 SNP were finally evaluated (Supplementary Table 1). Univariate analysis showed a significant association at a 5%-alpha level between the 7 of 83 SNP and the occurrence of CNS metastasis: AKT1-rs3803304, AKT2-rs3730050, AKT2-rs8100018, PDK1-rs11686903, PDK1-rs11904366, PI3KR1-rs251408 and PI3KR1-rs706716 (Table 2). After Bonferroni correction (p-value<0.00085), only PI3KR1-rs706716 remained significantly associated with CNS metastasis. For the multivariate analysis including SNP associated with occurrence of CNS metastasis in univariate analysis at the 5%-alpha level, we excluded AKT2-rs8100018 since it had an identical distribution as AKT2-rs370050 in our population. Multivariate analysis confirmed an association between AKT1-rs3803304 CC, AKT2-rs3730050 AA, PDK1-rs11686903 TT and PI3KR1-rs706716 TT and the occurrence of CNS metastases (Table 2, Figure 3) with similar strength of association. When these 4 SNP were combined into a score, three prognostic groups could be identified: five-year cumulative incidence of CNS metastasis rate were 34.8%, 68.9% and 85.7% for patients with a score of 0 (no risk genotype), 1 (1 risk genotype) and 2 (2 risk genotypes). The score was significantly associated with the occurrence of CNS metastasis, with and without adjustment for the significant clinical parameters, hormone receptor status and HER2 status and peritumoral emboli (Table 3).

Confidence intervals of sub-distribution hazard ratios were re-estimated using a bootstrap approach and were close to the results previously obtained. The C index of Harrell, evaluating the discriminant capacity of the score was 0.607 (95%CI: 0.557-0.657). The interaction test between the score and clinical parameters was not significant for both hormone receptors status and HER2 status, and peritumoral emboli (Supplementary Table 3).

DISCUSSION

CNS metastases are a frequent and life-threatening complication of metastatic breast cancer that not only limits survival, but induces morbidity and greatly impairs quality of life. The established risk factors of CNS metastasis such as HER2-positive and triple-negative tumor status [1, 9-14, 32] were confirmed in the present cohort. Interestingly, we observed that peritumoral emboli were also associated with increased risk of CNS metastasis (Table 1, Figure 2E). We sought to explore variations of PI3K-AKT-mTOR pathway genes for association with increased risk of CNS metastasis in metastatic breast cancer. AKT1-rs3803304, AKT2-rs3730050, AKT2-rs8100018, PDK1-rs11686903, PDK1-rs11904366, PI3KR1-rs251408 and PI3KR1-rs706716 were associated with risk of CNS metastasis in univariate analysis at a 5% alpha level, however, after Bonferroni correction, only PI3KR1-rs706716 remained significantly associated (Table 2). Multivariate analysis confirmed associations between AKT1-rs3803304, AKT2-rs3730050, PDK1-rs11686903 and PI3KR1-rs706716, and the risk of CNS metastasis. The risk was at least double for patients with AKT1-rs3803304 (CC), AKT2-rs3730050 (AA), PDK1-rs11686903 (TT)

and PI3KR1-rs706716 (TT). The combination of SNP into a score enhanced the predictive power (Table 3).

None of these SNP has previously been reported in studies on CNS metastases or breast cancer. PI3KR1-rs706716 and PDK1-rs11686903 have not been associated with other diseases. AKT1-rs3803304 was associated with a lower risk of death in 45 patients with recurrent or initially metastatic head and neck squamous cell carcinoma [33] and a better response to treatment was associated with this SNP in 45 esophageal cancer patients with adenocarcinoma or squamous cell carcinoma who had undergone chemoradiotherapy and surgery [34]. AKT2-rs3730050 was associated with shorter survival in 319 patients with muscle-invasive and metastatic bladder cancer [35].

An analysis of 16 SNP of 5 genes of the PI3K-AKT-mTOR pathway (PIK3CA, PTEN, AKT1, AKT2, FRAP1) and occurrence of brain metastases in 317 non-small cell lung cancer patients showed that AKT1-rs2498804, AKT1-rs2494732 and PIK3CA-rs2699887 increased the risk of brain metastases at 24-months follow-up [36]. None of these SNP were identified as at risk of CNS metastases here.

How precisely SNP in a given pathway modulate course of disease in cancer patients remains to be elucidated. PI3KCA mutations are the most common mutations of the PI3K pathway in breast cancer, depending on the subtype of cancer, with the lowest rate in triple negative cancer. The prognostic role of PI3KCA mutations remains controversial, but they tend to be associated with more favorable outcomes [28]. Other alterations of the PI3K-AKT-mTOR pathway in breast cancer include PI3KR1 mutations (3%), decreased PI3KR1 expression (62%), AKT1 mutations (3%) and overexpression (25.3%), PTEN mutations (2-12.5%), and PTEN loss (28%) [20, 28, 37]. An alteration of at least one parameter of PI3K pathway has been reported in

72% of tumors [37]. Among 52 breast cancer brain metastases and 12 matched primary breast cancers, expression of p-AKT and p-S6, and lack of PTEN expression were found in 75%, 69% and 25% of brain metastases. Concordances rates between primary tumor and brain metastases were 67% for p-AKT expression, 58% for p-S6 expression and 83% for PTEN [30]. Given this high prevalence of pathway mutations, it is conceivable that SNP modulate tumor cell-intrinsic behavior. Yet, it cannot be excluded that the SNP also determine how the host`s microenvironment interacts with metastatic tumor cells.

Limitations of this study include the lack of an appropriate validation cohort and the lack of systematic prospective patient assessment for CNS metastasis. A separate analysis for patients with brain as opposed to leptomeningeal metastases would have been interesting, but was not feasible for lack of statistical power. Still, our results support the notion that the identification of risk factors for CNS metastasis may enable screening and earlier detection when numerous therapeutic options are still available, with the consequences of limiting neurological impairment and preserving quality of life longer. Such risk profiles may also facilitate the development of new strategies of prevention in populations at risk.

TABLES TITLES AND FIGURES LEGENDS

Tables

Table 1: Patient characteristics

Table 2: Association between SNP and CNS metastases in univariate analysis, after Bonferroni correction, and in multivariate analysis

Table 3: Combination of SNP and score

Figure legends

Figure 1. **CONSORT diagram.** Patients with CNS metastases include patients with brain or leptomeningeal metastasis, patients without CNS metastases are defined as patients with extra-cerebral metastases without any neurological symptoms or signs, with normal brain MRI before death or during 5-years follow-up after diagnosis of first metastasis.

Figure 2. **Clinical correlates of CNS metastasis and outcome.** A,B,C. OS from breast cancer diagnosis (A), first diagnosis of metastasis (B) and CNS metastasis diagnosis (C). D.E. Cumulative incidence of CNS metastasis after the diagnosis of first metastasis by hormonal and HER2 status (D) or absence or presence of tumoral vascular emboli (E).

Figure 3. **SNP linked to CNS metastasis.** Cumulative incidence of CNS metastases after diagnosis of first metastasis for SNP significantly associated with CNS metastases on multivariate analysis. A: AKT1-RS3803304, B: PDK1-RS11686903, C: AKT2-RS370050, D: PI3KR1-RS706716, E: score

Supplementary material

Supplementary Note 1: Demographic and clinical data collected for the analysis

Supplementary Note 2: Details on genomic analyses and SNP studies

Supplementary Table 1: Genes and Single Nucleotide Polymorphism selected for this study

Supplementary Table 2: No association between initial treatment of breast cancer and risk of CNS metastases

Supplementary Table 3: Interaction test between the score and clinical parameters

References

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Identification of single nucleotide polymorphisms of the PI3K-AKT-mTOR pathway as a risk factor of central nervous system metastasis in metastatic breast cancer

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Abstract

Introduction: The PI3K-AKT-mTOR pathway may be involved in the development of CNS metastasis from breast cancer. Accordingly, here we explored whether single nucleotide polymorphisms (SNP) of this pathway are associated with altered risk of CNS metastasis formation in metastatic breast cancer patients.

Methods: The GENEOM study (NCT00959556) included blood sample collection from breast cancer patients treated in the neoadjuvant, adjuvant or metastatic setting. We identified patients with CNS metastases for comparison with patients without CNS metastasis, defined as absence of neurological symptoms or normal brain MRI before death or during 5-year follow-up. Eighty-eight SNP of PI3K-AKT-mTOR pathway genes were selected for analysis: AKT1 (17 SNP), AKT2 (4), FGFR1 (2), mTOR (7), PDK1 (4), PI3KR1 (11), PI3KCA (20), PTEN (17), RPS6KB1 (6).

Results: Of 342 patients with metastases, 207 fulfilled the inclusion criteria: 107 patients remained free of CNS metastases at last follow-up or date of death whereas 100 patients developed CNS metastases. Among clinical parameters, hormonal and HER2 status as well as tumor vascular emboli were associated with risk of CNS metastasis. Only PI3KR1-rs706716 was associated with CNS metastasis in univariate analysis after Bonferroni correction ($p<0.00085$). Multivariate analysis showed associations between AKT1-rs3803304, AKT2-rs3730050, PDK1-rs11686903 and PI3KR1-rs706716 and CNS metastasis.

Conclusion: PI3KR1-rs706716 may be associated with CNS metastasis in metastatic breast cancer patients and could be included in a predictive composite score to detect early CNS metastasis irrespective of breast cancer subtype.

Keywords

SNP, PTEN, brain, cerebral, leptomeningeal, prediction, genetic, predisposition, prevention

Text

INTRODUCTION

Breast cancer represents the most common cancer in women. Central nervous system (CNS) metastases occur in up to 10% of patients [1] and herald poor outcome: survival varies from 2.7 to 26.8 months with solid brain metastases, by breast cancer subtype [2] and is 4 months with leptomeningeal metastases [3-6]. Treatment of CNS metastasis aims not only for prolonging survival, but also at prevention or delay of neurological deterioration [7].

The identification of patients at risk could help to increase the efficacy of treatment of CNS metastasis. While cerebrospinal imaging is not part of standard follow-up in patients without neurological signs, the identification of subgroups of patients at risk could allow the implementation of more intensive follow-up and early intervention strategies.

Brain metastases risk is increased in triple-negative and human epidermal growth factor receptor (HER) 2-positive tumors [1, 8-14]. Risk factors for leptomeningeal metastases include opening of the ventricular system during surgery for solid brain metastases and resection of cerebellar metastases [15,16] and breast cancer patients specifically lobular subtype and triple-negative tumors [4,5,6,17].

Genetic variations could also help to define populations at risk. Single nucleotide polymorphisms (SNP) represent the most frequent type of variations of the human

genome [18]: they represent a single nucleotide variation at a specific position in the genome present at a frequency of 1%-50% in the general population that is maintained through heredity. While not causing disease, SNP can modify protein structure and function and thereby influence susceptibility to disease, including cancer [18].

The PI3K-AKT-mTOR pathway controls cell cycle, survival, differentiation, proliferation, motility, metabolism, and genomic stability and may be the most frequently activated pathway in human cancer [19,20,21]. Moreover, it also regulates the behavior of normal cells and contributes to host cell tumor cell interactions, e.g., during angiogenesis and inflammation [21-27]. PI3K-AKT-mTOR pathway genetic lesions are frequent in breast cancer and may mediate resistance to HER2-targeted agents and hormonal agents [28]. Activation of the PI3K pathway has specifically been observed in brain metastases from breast cancer, regardless of subtype as defined by hormone receptor or HER2 status [29,30], potentially mediated by the loss of PTEN expression as demonstrated in paired primary tumor and brain metastasis samples [31]. In fact, the loss of PTEN may directly promote brain invasiveness of metastatic breast cancer cells [27]. Here we sought to identify SNP of the PI3K-AKT-mTOR pathway associated with increased risk of CNS metastasis formation in patients with metastatic breast cancer.

MATERIALS AND METHODS

Patients

We conducted a secondary analysis in a subpopulation of patients from the GENEOM study (NCT00959556) that aimed at identifying constitutional genetic

variants predictive of response to chemotherapy and hormone therapy in adult patients with histologically confirmed breast cancer and included 914 women between November 2007 and January 2012. Our aim was to identify biomarkers of CNS metastasis risk among patients who developed metastases, at diagnosis of breast cancer or during follow-up (n=342) (Supplementary Note 1). Among patients fulfilling the inclusion criteria (n=207), CNS metastases were diagnosed at initial diagnosis of breast cancer or during follow-up, on brain MRI (n=87, 87%) or cranial CT (n=13, 13%). Histological confirmation was obtained in 10 brain metastasis patients. Leptomeningeal metastasis was defined by the presence of tumor cells in the cerebrospinal fluid (CSF) (n=25) or by characteristic MRI findings in a patient presenting with clinical signs suggestive of leptomeningeal metastases in the absence of positive CSF cytology (n=15). In 9 patients, the diagnosis was based on clinical evaluation and brain imaging only. Patients with extra-CNS metastasis only were defined by the absence of neurological symptoms or signs or normal brain MRI before death or during at least 5 years of follow-up after the diagnosis of the first metastasis. Patients with neurological symptoms of unclear origin, unclear cause of death, or follow-up below 5 years after the diagnosis of the first metastasis were excluded.

SNP selection

SNP of the PI3K-AKT-mTOR pathway were selected based on a systematic literature search. Eligible SNP had to have a minor allele frequency ≥ 0.05 in a European population, based on a 1000 genomes database (https://phase3browser.1000genomes.org/index.html). Eighty-eight SNP of the PI3K-AKT-mTOR pathway were finally considered for the genomic analysis. Two were

excluded (AKT1 rs3803304 and AKT1-rs2498786) due to a discordance between frequencies observed in our population and the database. A total of 86 SNP was finally analyzed (Supplementary Table 1). Details on genomic analyses and SNP studies are provided in Supplementary Note 2.

Statistical analysis

Overall survival (OS) was defined as time interval from diagnosis until death from any cause using the Kaplan-Meier method. Patients alive were censored at last follow-up. Cumulative incidence of CNS metastases was estimated using a competing risk approach considering the time interval from diagnosis of first metastasis to the date of diagnosis of CNS metastases, with death without CNS metastases considered as a competing event; patients alive without CNS metastases were censored at the date of last follow-up. Associations between CNS metastases and clinical parameters and genomic parameters were evaluated using sub-distribution hazard ratios estimated in Fine and Gray regression models. The first step consisted in the analysis of clinical factors. A multivariate competing risk regression model was performed for parameters significantly associated with CNS metastasis in univariate analysis ($p<0.05$).

A similar type of modeling was used for the second step of the analysis, evaluating the association of genomic parameters with the risk of CNS metastases. A SNP was considered as evaluable after verification of comparison for genotypic frequencies according to the Hardy-Weinberg equilibrium. SNP were excluded if the minor allele frequency was below 1%, or if genotyping analyses were performed in less than 90% of the patients of the cohort. Patients with less than 90% of SNP analyzed were excluded. For each SNP, the analysis was performed considering a dominant model

(dominant genotype versus others), recessive model (recessive genotype versus others) or log-additive model (3 ordered genotypic classes). The significance level was set to $p<0.05$, and a Bonferroni correction for multiple testing was also determined according to the number of independent SNP evaluated (threshold=0.05/number of independent SNP with $r^2<0.8$). SNP significantly associated with CNS metastasis in univariate analysis (using level $p<0.05$) were included in a multivariate competitive risks regression model including a step by step selection procedure of variables. Akaike information criterion (AIC) was applied for the selection of the appropriate SNP coding when several modellings were associated with CNS metastasis. A composite score was also computed from the estimated regression coefficients of the multivariate model including all SNP significant at a 5%-significant level. Lastly, the association of this genomic score with the risk of CNS metastases was evaluated in multivariate analysis adjusted for clinical parameters significantly linked to CNS metastasis. Confidence intervals were re-estimated using a boostrap approach with 1000 samplings. Harrell C discrimination index was computed. Statistical analyses were performed using Stata software (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP) and SNPassoc package of R software v3.3.1.

RESULTS

Clinical patient characteristics

Among the 914 patients enrolled in GENEOM, 342 patients had metastatic disease, 119 at diagnosis of breast cancer and 223 during follow-up. Among these 342 patients, 135 patients were excluded, leading to a study population of 207 patients (Figure 1). The median follow-up of this cohort is 9.1 years (range 4.8-10.5) after the

diagnosis of first metastasis. Overall, 107 patients remained free of CNS metastases at last follow-up or date of death whereas 100 patients developed CNS metastases. Median time interval between first diagnosis of metastasis and first diagnosis of brain metastasis was 1.4 years (0-8.4); it was 2.1 years between first diagnosis of metastasis and first diagnosis of leptomeningeal metastasis. CNS metastasis was the first metastatic site in 8 patients (8%). Treatment of brain metastases included surgery in 10, stereotactic radiotherapy in 14, whole brain radiotherapy in 51, and pharmacotherapy in 53 patients. Treatment of leptomeningeal disease included intra-cerebrospinal fluid pharmacotherapy in 28, focal radiotherapy in 5 and whole brain radiotherapy in 1, and systemic pharmacotherapy in 29 patients. At last follow-up, 189 patients had died. Median overall survival was 5.3 years (95% CI 4.5-6.1) since breast cancer diagnosis and 2.8 years (95% CI 2.3-3.3) since first diagnosis of metastasis. Median overall survival after diagnosis of CNS metastasis was 4.7 months (95% CI: 3.6-6.4) for brain metastases patients and 4 months (95% CI: 2.3-4.9) for leptomeningeal metastases patients (Figure 2A,B,C).

Patient characteristics and association of clinical characteristics with risk of CNS metastases are summarized in Table 1. Median age at breast cancer diagnosis was 50 years (range, 22 to 79); age was not associated with occurrence of CNS metastasis. A lower time interval between breast cancer diagnosis and diagnosis of first metastasis was associated with an increased risk of CNS metastases whereas histology and Scarff Bloom Richardson scores were not. Cumulative incidence of CNS metastases was higher in patients with hormone receptor-positive and HER2-positive or triple-negative tumors, as well as in patients with peritumoral emboli on univariate and multivariate analysis (Figure 2D,E). Initial treatment was not associated to the occurrence of CNS metastases (Supplementary Table 2).

SNP analyses

Of 86 SNP, 3 were excluded from analysis: RPS56KB1-rs1292033 for not respecting the Hardy Weinberg equilibrium, and PI3KCA-rs17849071 and PI3KCA-rs7641889 because 10.1% of patients could not be genotyped for these 2 SNP. No SNP had a minority allele frequency below 1%. The genotype of all patients was analyzed for more than 90% of SNP, and thus all patients were included in this analysis. Thus 83 SNP were finally evaluated (Supplementary Table 1). Univariate analysis showed a significant association at a 5%-alpha level between the 7 of 83 SNP and the occurrence of CNS metastasis: AKT1-rs3803304, AKT2-rs3730050, AKT2-rs8100018, PDK1-rs11686903, PDK1-rs11904366, PI3KR1-rs251408 and PI3KR1-rs706716 (Table 2). After Bonferroni correction (p-value<0.00085), only PI3KR1-rs706716 remained significantly associated with CNS metastasis. For the multivariate analysis including SNP associated with occurrence of CNS metastasis in univariate analysis at the 5%-alpha level, we excluded AKT2-rs8100018 since it had an identical distribution as AKT2-rs370050 in our population. Multivariate analysis confirmed an association between AKT1-rs3803304 CC, AKT2-rs3730050 AA, PDK1-rs11686903 TT and PI3KR1-rs706716 TT and the occurrence of CNS metastases (Table 2, Figure 3) with similar strength of association. When these 4 SNP were combined into a score, three prognostic groups could be identified: five-year cumulative incidence of CNS metastasis rate were 34.8%, 68.9% and 85.7% for patients with a score of 0 (no risk genotype), 1 (1 risk genotype) and 2 (2 risk genotypes). The score was significantly associated with the occurrence of CNS metastasis, with and without adjustment for the significant clinical parameters, hormone receptor status and HER2 status and peritumoral emboli (Table 3).

Confidence intervals of sub-distribution hazard ratios were re-estimated using a bootstrap approach and were close to the results previously obtained. The C index of Harrell, evaluating the discriminant capacity of the score was 0.607 (95%CI: 0.557-0.657). The interaction test between the score and clinical parameters was not significant for both hormone receptors status and HER2 status, and peritumoral emboli (Supplementary Table 3).

DISCUSSION

CNS metastases are a frequent and life-threatening complication of metastatic breast cancer that not only limits survival, but induces morbidity and greatly impairs quality of life. The established risk factors of CNS metastasis such as HER2-positive and triple-negative tumor status [1, 9-14, 32] were confirmed in the present cohort. Interestingly, we observed that peritumoral emboli were also associated with increased risk of CNS metastasis (Table 1, Figure 2E). We sought to explore variations of PI3K-AKT-mTOR pathway genes for association with increased risk of CNS metastasis in metastatic breast cancer. AKT1-rs3803304, AKT2-rs3730050, AKT2-rs8100018, PDK1-rs11686903, PDK1-rs11904366, PI3KR1-rs251408 and PI3KR1-rs706716 were associated with risk of CNS metastasis in univariate analysis at a 5% alpha level, however, after Bonferroni correction, only PI3KR1-rs706716 remained significantly associated (Table 2). Multivariate analysis confirmed associations between AKT1-rs3803304, AKT2-rs3730050, PDK1-rs11686903 and PI3KR1-rs706716, and the risk of CNS metastasis. The risk was at least double for patients with AKT1-rs3803304 (CC), AKT2-rs3730050 (AA), PDK1-rs11686903 (TT)

and PI3KR1-rs706716 (TT). The combination of SNP into a score enhanced the predictive power (Table 3).

None of these SNP has previously been reported in studies on CNS metastases or breast cancer. PI3KR1-rs706716 and PDK1-rs11686903 have not been associated with other diseases. AKT1-rs3803304 was associated with a lower risk of death in 45 patients with recurrent or initially metastatic head and neck squamous cell carcinoma [33] and a better response to treatment was associated with this SNP in 45 esophageal cancer patients with adenocarcinoma or squamous cell carcinoma who had undergone chemoradiotherapy and surgery [34]. AKT2-rs3730050 was associated with shorter survival in 319 patients with muscle-invasive and metastatic bladder cancer [35].

An analysis of 16 SNP of 5 genes of the PI3K-AKT-mTOR pathway (PIK3CA, PTEN, AKT1, AKT2, FRAP1) and occurrence of brain metastases in 317 non-small cell lung cancer patients showed that AKT1-rs2498804, AKT1-rs2494732 and PIK3CA-rs2699887 increased the risk of brain metastases at 24-months follow-up [36]. None of these SNP were identified as at risk of CNS metastases here.

How precisely SNP in a given pathway modulate course of disease in cancer patients remains to be elucidated. PI3KCA mutations are the most common mutations of the PI3K pathway in breast cancer, depending on the subtype of cancer, with the lowest rate in triple negative cancer. The prognostic role of PI3KCA mutations remains controversial, but they tend to be associated with more favorable outcomes [28]. Other alterations of the PI3K-AKT-mTOR pathway in breast cancer include PI3KR1 mutations (3%), decreased PI3KR1 expression (62%), AKT1 mutations (3%) and overexpression (25.3%), PTEN mutations (2-12.5%), and PTEN loss (28%) [20, 28, 37]. An alteration of at least one parameter of PI3K pathway has been reported in

72% of tumors [37]. Among 52 breast cancer brain metastases and 12 matched primary breast cancers, expression of p-AKT and p-S6, and lack of PTEN expression were found in 75%, 69% and 25% of brain metastases. Concordances rates between primary tumor and brain metastases were 67% for p-AKT expression, 58% for p-S6 expression and 83% for PTEN [30]. Given this high prevalence of pathway mutations, it is conceivable that SNP modulate tumor cell-intrinsic behavior. Yet, it cannot be excluded that the SNP also determine how the host`s microenvironment interacts with metastatic tumor cells.

Limitations of this study include the lack of an appropriate validation cohort and the lack of systematic prospective patient assessment for CNS metastasis. A separate analysis for patients with brain as opposed to leptomeningeal metastases would have been interesting, but was not feasible for lack of statistical power. Still, our results support the notion that the identification of risk factors for CNS metastasis may enable screening and earlier detection when numerous therapeutic options are still available, with the consequences of limiting neurological impairment and preserving quality of life longer. Such risk profiles may also facilitate the development of new strategies of prevention in populations at risk.

TABLES TITLES AND FIGURES LEGENDS

Tables

Table 1: Patient characteristics

Table 2: Association between SNP and CNS metastases in univariate analysis, after Bonferroni correction, and in multivariate analysis

Table 3: Combination of SNP and score

Figure legends

Figure 1. **CONSORT diagram.** Patients with CNS metastases include patients with brain or leptomeningeal metastasis, patients without CNS metastases are defined as patients with extra-cerebral metastases without any neurological symptoms or signs, with normal brain MRI before death or during 5-years follow-up after diagnosis of first metastasis.

Figure 2. **Clinical correlates of CNS metastasis and outcome.** A,B,C. OS from breast cancer diagnosis (A), first diagnosis of metastasis (B) and CNS metastasis diagnosis (C). D.E. Cumulative incidence of CNS metastasis after the diagnosis of first metastasis by hormonal and HER2 status (D) or absence or presence of tumoral vascular emboli (E).

Figure 3. **SNP linked to CNS metastasis.** Cumulative incidence of CNS metastases after diagnosis of first metastasis for SNP significantly associated with CNS metastases on multivariate analysis. A: AKT1-RS3803304, B: PDK1-RS11686903, C: AKT2-RS370050, D: PI3KR1-RS706716, E: score

Supplementary material

Supplementary Note 1: Demographic and clinical data collected for the analysis

Supplementary Note 2: Details on genomic analyses and SNP studies

Supplementary Table 1: Genes and Single Nucleotide Polymorphism selected for this study

Supplementary Table 2: No association between initial treatment of breast cancer and risk of CNS metastases

Supplementary Table 3: Interaction test between the score and clinical parameters

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TABLES

Table 1: Patient characteristics and association with risk of CNS metastases

			Univariate analysis (clinical factors)		Multivariate analysis (clinical factors)	
	N (%)	5-year cumulative incidence of CNS metastases (95% CI)	SHR (95% CI)	p-value	SHR (95% CI)	p-value
Age at breast cancer diagnosis (/year)		-	0.992 (0.976-1.010)	0.39	n.a.	n.a.
Time between breast cancer diagnosis and first diagnosis of metastasis (/month)		-	0.994 (0.991-0.998)	**0.007**	0.995 (0.989-1.000)	0.051
Histology						
• Infiltrative ductal carcinoma	155 (75.2%)	45.1% (37.2-52.8)	1			
• Infiltrative lobular carcinoma	19 (9.2%)	21.1% (6.6-41.0)	0.36 (0.13-1.001)	0.06	n.a.	n.a.
• Other	32 (15.5%)	53.1% (34.7-68.5)	1.44 (0.95-2.20)	0.09	n.a.	n.a.
SBR grade						
• I	14 (6.8%)	28.6% (8.8-52.4)	1			
• II	109 (52.7%)	43.1% (33.7-52.2)	1.93 (0.70-5.37)	0.21	n.a.	n.a.
• III	49 (23.7%)	59.2% (44.2-71.4)	2.70 (0.95-7.68)	0.06	n.a.	n.a.
• Other (non gradable/ not avalaible)	35 (16.9%)	30.4% (13.5-49.3)	1.43 (0.44-4.62)	0.55	n.a.	n.a.
HR and HER2 status						
• Hormone receptor-positive, HER2-negative	108 (56.5%)	35.2% (26.3-44.2)	1		1	
• Hormone receptor-positive, HER2-positive	33 (17.3%)	51.5% (33.5-66.9)	1.77 (1.06-2.96)	**0.028**	1.70 (1.03-2.79)	**0.037**
• Hormonel receptor-negative, HER2-positive	24 (12.6%)	58.3% (36.5-75.0)	1.81 (0.98-3.33)	0.057	1.32 (0.63-2.75)	0.45
• Triple-negative	26 (13.6%)	69.2% (47.8-83.3)	2.54 (1.43-4.51)	**0.001**	2.10 (1.16-3.79)	**0.014**
Peritumoral vascular emboli						
• No	127 (69.8%)	40.9% (32.4-49.3)	1		1	
• Yes	55 (30.2%)	58.2% (44.1-69.9)	1.78 (1.18-2.68)	**0.006**	1.83 (1.20-2.77)	**0.005**
Presence of metastatic sites:						
• at diagnosis or whithin 3 months after breast cancer diagnosis	71 (34.3%)	40.9% (29.4-51.9)	1			
• > 3 months after breast cancer diagnosis	136 (65.7%)	45.6% (37.1-53.7)	1.28 (0.83-1.96)	0.26	n.a.	n.a.
Metastastic sites at first diagnosis of metastases: Bone metastasis						
• No	91 (44.0%)	48.5% (37.8-58.1)	1			
• Yes	116 (56.0%)	40.5% (31.6-49.3)	0.75 (0.51-1.11)	0.15	n.a.	n.a.
Lung metastasis						
• No	141 (68.1%)	41.1% (33.0-49.1)	1			

• Yes	66 (31.9%)	50.0% (37.5-61.3)	1.30 (0.87-1.95)	0.20	n.a.	n.a.
Pleura metastasis • No • Yes	 190 (91.8%) 17 (8.2%)	 43.7% (36.6-50.6) 47.1% (23.0-68.0)	 1 1.08 (0.49-2.39)	 0.85	 n.a.	 n.a.
Mediastinum metastasis • No • Yes	 156 (75.4%) 51 (24.6%)	 43.0% (35.1-50.5) 47.1% (33.0-59.9)	 1 1.13 (0.72-1.78)	 0.58	 n.a.	 n.a.
Liver metastasis • No • Yes	 121 (58.5%) 86 (41.5%)	 41.3% (32.5-49.9) 47.7% (36.8-57.7)	 1 1.14 (0.77-1.70)	 0.51	 n.a.	 n.a.
Peritoneum metastasis • No • Yes	 199 (96.1%) 8 (3.9%)	 44.7% (37.7-51.5) 25.0% (3.7-55.8)	 1 0.50 (0.10-2.18)	 0.33	 n.a.	 n.a.
Cutaneous metastasis • No • Yes	 178 (86.0%) 29 (14.0%)	 42.7% (35.4-49.8) 51.7% (32.5-67.9)	 1 1.27 (0.74-2.18)	 0.38	 n.a.	 n.a.
Loco-regional metastasis • No • Yes	 117 (56.5%) 90 (43.5%)	 38.5% (29.7-47.2) 51.1% (40.4-60.9)	 1 1.35 (0.92-2.00)	 0.13	 n.a.	 n.a.
Other metastasis • No • Yes	 185 (89.4%) 22 (10.6%)	 42.2% (35.0-49.2) 59.1% (36.1-76.2)	 1 1.72 (0.88-3.33)	 0.11	 n.a.	 n.a.

CNS : central nervous system; SBR: Scarff Bloom Richardson
SHR: Sub-distribution hazard ratio estimated in Fine and Gray model
Multivariate analysis included: HR and HER2 status, peritumoral vascular emboli, time between breast cancer diagnosis and first diagnosis of metastasis.
n.a.: not applicable because not included in multivariate regression model ($p>0.05$ in univariate analysis)

Table 2: Association between SNP and CNS metastases in univariate analysis, after Bonferroni correction, and in multivariate analysis, for the 7 SNP with p-values <0.05 on univariate analysis

			Univariate analysis (SNP)				Multivariate analysis (SNP)		
SNP	N	5-year cumulative incidence of CNS metastases (95% CI)	SHR (95% CI)	p-value	AIC	Significance After Bonferroni correction (0.00085)	Regression coefficient	SHR (95% CI)	p-value
AKT1-RS3803304									
CG-GG	198	42.9% (36.0-49.7)	1					**1**	
CC (recessive**)**	9	66.7% (28.2-87.8)	2.17 (1.06-4.42)	0.033	1003.0	NS	**1.00**	**2.72 (1.30-5.68)**	**0.008**
AKT2 - RS3730050									
AG-GG	189	41.8% (34.7-48.7)	1					**1**	
AA (recessive)	18	66.7% (40.4-83.4)	2.07 (1.06-4.02)	0.033	1001.5	NS	**0.73**	**2.06 (1.03-4.14)**	**0.041**
AKT2 - RS8100018									
CG-GG	189	41.8% (34.7-48.7)	1						
CC (recessive)	18	66.7% (40.4-83.4)	2.07 (1.06-4.02)	0.033	1001.5	NS	ND		
PDK1-RS11686903									
CC-CT	184	40.2% (33.1-47.2)	1					**1**	
TT (recessive)	23	73.9% (50.9-87.3)	2.35 (1.37-4.02)	0.002	997.7	NS	**0.87**	**2.38 (1.40-4.05)**	**0.001**
Log-additive :			1.57 (1.13-2.18)	0.007	998.3	NS			
CC	75	36.0% (25.3-46.8)							
CT	109	43.1% (33.7-52.2)							
TT	23	73.9% (50.9-87.3)							
PDK1-RS11904366									
GT-TT	68	32.4% (21.7-43.5)	1						
GG (dominant)	139	49.6% (41.1-57.6)	1.67 (1.06-2.63)	0.028	1000.8	NS	NS*		
Log-additive :			1.57 (1.04-2.39)	0.033	1000.9	NS			
GG	139	49.6% (41.1-57.6)							
GT	62	33.9% (22.5-45.6)							
TT	6	16.7% (0.8-51.7)							
PI3KR1-RS251408									
AA-AG	173	40.5% (33.1-47.7)	1						
GG (recessive)	34	61.8% (43.4-75.7)	1.62 (1.03-2.54)	0.035	1002.5	NS	NS*		
PI3KR1-RS706716									
CC-CT	198	41.9% (35.0-48.7)	1					**1**	
TT (recessive)	9	88.9% (43.3-98.4)	3.16 (1.71-5.87)	0.0003	999.0	S	**0.88**	**2.42 (1.12-3.25)**	**0.025**

SHR: Sub-distribution hazard ratio estimated in Fine and Gray model
Univariate analysis : NS : non significant, S : significant after Bonferroni correction for multiple testing
The backward stepwise multivariate regression model included : AKT1-RS3803304, AKT2 - RS3730050, PDK1-RS11686903 (as recessive), PDK1-RS11904366 (as dominant), PI3KR1-RS251408, and PI3KR1-RS706716. PDK1-RS11686903 and PDK1-RS11904366 were included as recessive and dominant because of a lower AIC obtained in univariate analysis than with log-additive model.

ND : not done : AKT2 - RS8100018 was not included in multivariate analysis because of its identical distribution with AKT2 - RS3730050 in our population
NS*: SNPs that were not significant and removed from backward stepwise multivariate regression model (p-value=0.15 for PDK1 - RS11904366 and p-value=0.38 for PI3KR1 - RS251408).

Table 3 : Combination of SNP and score

			Original hazard ratio				Boostrap analysis			
	Number of patients	5-year cumulative incidence of CNS metastases (95% CI)	SHR (95% CI)	p-value	SHR (95% CI) adjusted for clinical parameters*	p-value	SHR (95% CI)	p-value	SHR (95% CI) adjusted for clinical parameters*	p-value
SCORE 0	155	34.8% (27.4-42.3)	1		1		1		1	
SCORE 1	45	68.9% (53.2-80.3)	2.55 (1.65-3.94)	<0.001	2.07 (1.30-3.30)	0.002	2.55 (1.60-4.07)	<0.001	2.07 (1.26-3.42)	0.004
SCORE 2	7	85.7% (33.4-97.9)	4.62 (1.82-11.7)	0.001	3.82 (1.39-10.5)	0.009	4.62 (1.53-13.9)	0.007	3.82 (0.40-36.9)	0.25
SCORE (continuous value)			2.32 (1.64-3.30)	<0.001	2.01 (1.37-2.95)	<0.001	2.32 (1.61-3.36)	<0.001	2.01 (1.34-3.03)	0.001

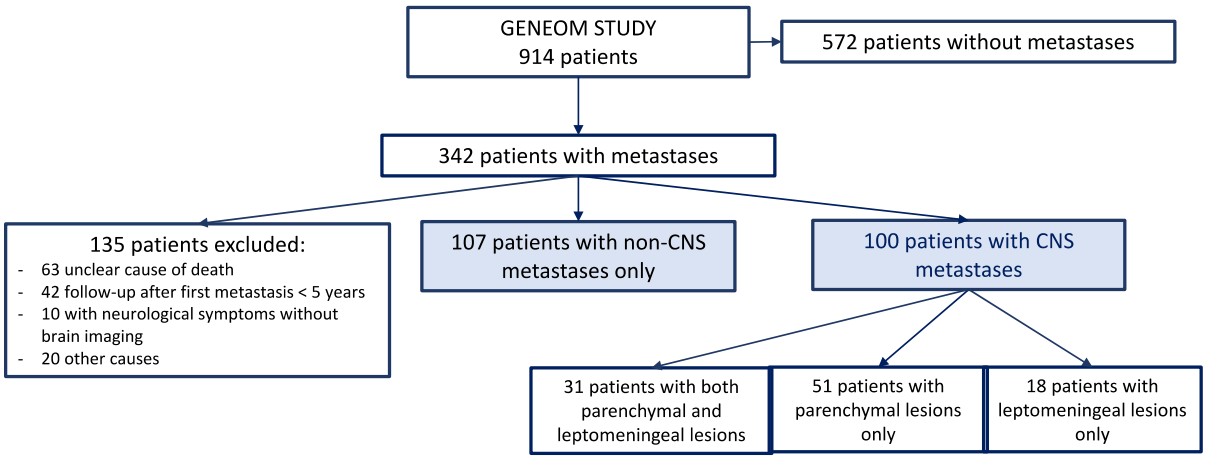
SHR: Sub-distribution hazard ratio estimated in Fine and Gray model

* Adjustment for estrogen and progesterone receptors, HER2 status, peritumoral emboli

Figure

FIGURES

Figure 1



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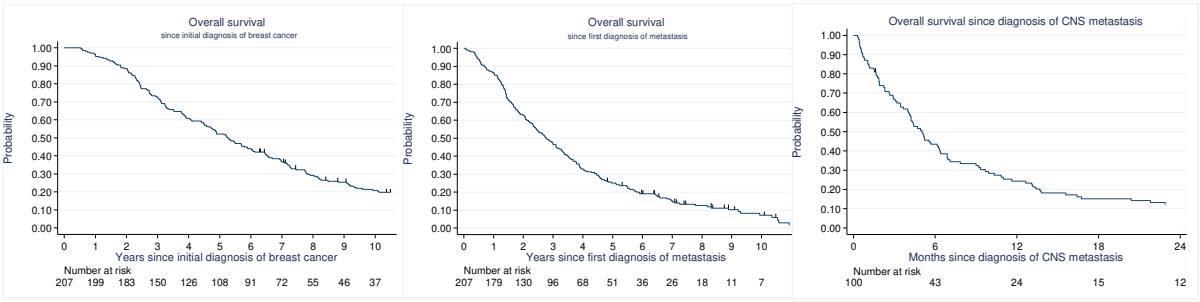
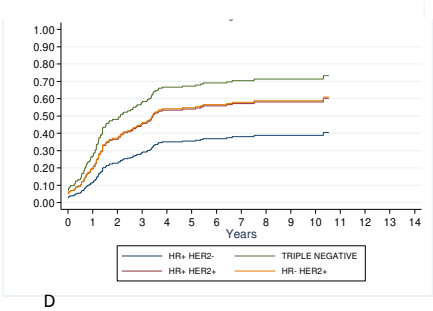




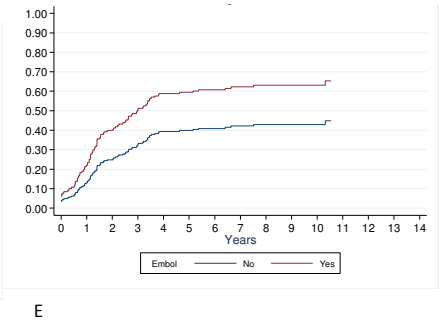
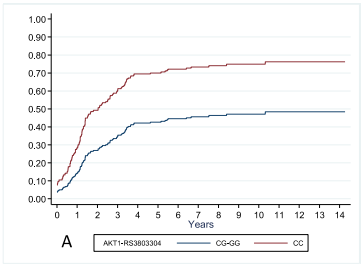


Figure 3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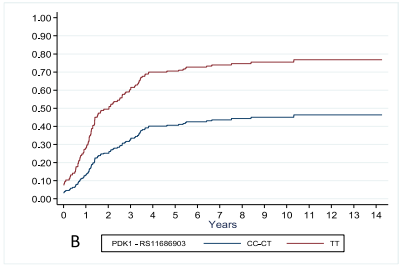
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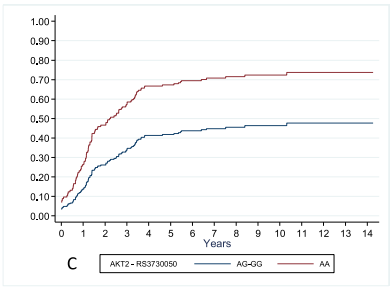
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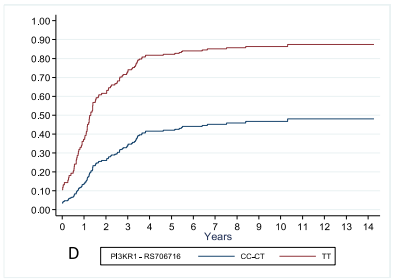
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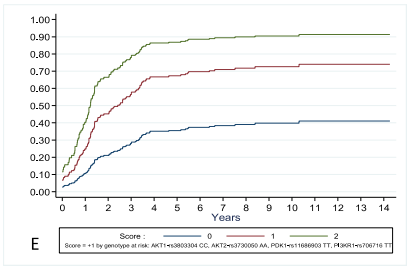
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Conflict of interest:

ELR has received research grants from Mundipharma, Amgen and honoraria for lectures from Mundipharma and Novartis.

NB has no conflict of interest to declare.

AD has no conflict of interest to declare.

ET has no conflict of interest to declare.

MCLD has no conflict of interest to declare.

AM has no conflict of interest to declare.

MP has received research support from Böhringer-Ingelheim, GlaxoSmithKline, Merck Sharp & Dome and Roche and honoraria for lectures, consultation or advisory board participation from Bristol-Myers Squibb, Novartis, Gerson Lehrman Group (GLG), CMC Contrast, GlaxoSmithKline, Mundipharma, Roche, Astra Zeneca and AbbVie.

MW has received research grants from Acceleron, Actelion, Bayer, Isarna, Merck, Sharp & Dohme, Merck (EMD, Darmstadt), Novocure, OGD2, Piqur and Roche and honoraria for lectures or advisory board participation or consulting from Abbvie, BMS, Celldex, Immunocellular Therapeutics, Magforce, Merck, Sharp & Dohme, Merck (EMD, Darmstadt), Novocure, Pfizer, Roche, Teva and Tocagen.

FR has no conflict of interest to declare.

JB has no conflict of interest to declare.

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